The Natural History of Gene Exchange Among Bacteria

Progress Report for Dr. J. Lederberg

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1 The Natural History of Gene Exchange among Bacteria

Several intriguing reports have appeared recently which indicate that unrelated species exchange genetic information. Most of this work involved enteropathogenic species and has lead to widespread anxiety concerning the hazards of laboratory 'genetic engineering'. The basic hypothesis underlying my thesis research is that DNA transfer is ubiquitous in nature, and bacteria commonly ignore our taxonomic classifications. The demonstration that plasmids from **Staphylococcus aureus** can replicate and express their genetic information in **Bacillus subtilis** (Ehrlich, S.D. (1977) P.N.A.S. USA **74**:1680) generated much of the intellectual impetus for this project. It led us to think that soil may be the best place to look for occurrences of gene transfer of the type described.

2 Progress Report

I am currently screening bacterial isolates from several soil samples for the presence and transfer of plasmids. The focus is on antibiotic-resistance genes because of their well known promiscuity and easily selectable phenotype. Genetic and biochemical characterization of these plasmids may provide insights into the frequency and mechanism of gene transfer between widely divergent species.

Preliminary evidence has shown that an identical plasmid (by the criterion of multiple restriction enzyme digests and agarose gel electrophoresis) exists in several independent isolates from a single soil sample. Partial identification of these species places them into three distinct phenotypic classes. They are all **Pseudomonad** sp. Studies are in progress to discover the function of these plasmids in the cell. Their transfer to **Escherischia coli** and **B. subtilis** by transformation or conjugation was so far unsuccessful. Another soil sample gave a much wider spectrum of strain variation. A collection of about 200 isolates is being screened for plasmids. We hope to demonstrate homologies between isolates at the DNA level and to experimentally show transfer of genetic information to other isolates or standard reference strains.

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1 Introduction

This review summarizes the major aspects of my research from October 1977 to the present. I will attempt to specify the nature of the problem under study, present some background on the history of the domain and my interest in it, and show some preliminary results from a series of initial experiments. Finally, there will be a summary of current efforts and some speculations on where this may lead in the near future.

1.1 Task Domain

The fundamental hypothesis that motivates my thesis research is that bacteria in nature commonly exchange genetic information laterally across species lines. A class of genes particularly promiscuous in nature [1] and also easily amenable to experimental manipulation is the antibiotic-resistance determinants. Several investigators documented transfer of R-factor-coded antibiotic-resistance genes from one species to another [2][3].

The major implication is that bacterial evolution is vastly accelerated under natural conditions by the presence and transfer of plasmids and phages. Reanney emphasized the importance of the ubiquity of extrachromosomal elements by asserting, "No single organism can evolve in genetic isolation from other organisms with which it shares a common environment" ([4],p565). This leads to important questions concerning the natural host ranges of transferable genetic agents.

At present, the best documented cases of interspecies transfer of plasmids involve the pathogenic microflora [7]. Some of the most promiscuous plasmids belong to incompatibility group P-1, and were originally isolated in carbenicillin-resistant Pseudomonas aeruginosa. Other P-1 group plasmids have been found in natural isolates of Klebsiella aerogenes, Serratia marcescens, and Bordetella bronchiseptica. Stanisich and Ortiz [5] presented evidence that these plasmids all derived from a common ancestor, despite their occurrence in diverse taxa in various parts of the world. Subsequently, it was shown that P-1 plasmids can be transferred into Agrobacterium, Azotobacter, Acinetobacter, Chromobacterium, Escherichia, Erwinia, Nisseria, Proteus, Rhizobium, Rhodopseudomonas, Rhodospirillum, Shigella, and Vibrio [6].

The demonstration that plasmids from Staphylococcus aureus can replicate and express their genetic information in Bacillus subtilis [8] generated much of the intellectual impetus for this project. It led us to think that soil may be the best place to look for new occurrences of gene transfer of the type described.

1.2 Historical Background on the 'Soil Angle'

I am primarily interested in gene exchange among soil bacteria. In addition to the B. subtilis demonstration, an early study on the prevalence of antibiotic-resistance in "drug-free" communities gave a very interesting result. Gardner et.al. [9] screened stool specimens and soil samples from an isolated community in the South Pacific. They found two out of forty specimens containing R-factors; one from the bowel of a native and the other from an 'alcaligenes-like' species of the soil. As Falkow points out ([1],p. 224), most studies focussed on factors involved in human and animal colonization, and little has been done on the examination of soil flora to assess the prevalence of R-factor-containing organisms.

Another motivation for studying antibiotic-resistance gene transfer among soil microorganisms is the suggestion of Walker and Walker [10] that antibiotic-producers may be the origin of, and reservoir for, antibiotic resistance genes. Benveniste and Davies [11] specifically tested this notion by comparing the antibiotic inactivating enzymes produced by actinomycetes and bacteria containing R-plasmids, and found them to be quite similar. More recent results from Davies' lab [12] failed to find homology at the DNA level between Streptomyces producing neomycin-modifying enzymes and R-plasmids purified from clinical isolates. Their conclusion: if gene transfer occurred, "it was a very long time ago, and probably passed through a number of unrelated genera en route", has been questioned [13].

1.3 Rationale

There are at least three general areas where a study of antibiotic resistance among soil microorganisms may have an impact. Primarily, scientific issues related to the occurrence of antibiotic-resistance gene exchange among soil microorganisms will be addressed. These include:

1) the frequency (or ubiquity) of antibiotic resistance in the soil. This is essentially a previously unanswered epidemiological question.

2) the host range of newly-isolated R-factors. What organisms can support the replication and expression of genes isolated from a given species? This is essentially the interspecies transfer of plasmids.

3) mechanisms of transfer. Can general ecological principles be derived from such studies? For example,

in a summary of genetic exchange across diverse taxa, Reanney [6] postulates that this ability is a function of cell-to-cell contact offered by their grouped ecology in a nutritionally favorable environment. Many genera which exchange genes exist within the rhizosphere; others within the mammalian gut. Known mechanisms of transfer include transformation, transduction, and conjugation.

4) the origin of R-factors, as alluded to in Section 1.2.

Secondly, practical applications of knowledge about the ecology of these plasmids may follow in areas such as widespread (and often unnecessary) prescription of antibiotics to human patients, antibiotics in cattle feed, and other dissemination and selection of these agents in the biosphere. These are very complex cost-benefit questions. For example, a physician must always weigh his patients' immediate benefit from antibiotic therapy against the cost to society as a whole from the selection of R-factors. Cattle ranchers can get 5% or 10% more beef from cattle fed with prophylactic antibiotics than without the drugs. Finland [14] and Jukes [15] review of some of these issues. More data on the prevalence and transfer of R-factors in the soil may be useful in such a debate.

Finally, there may be clear technological advances to be made during this study. New cloning vectors for Bacillus subtilis, or other bacteria, will possibly be found with desirable properties in a selective screening procedure. The staphylococcus plasmids have many of these properties, such as small size, few or single restriction enzyme sites, and easily selectable markers (antibiotic resistance), but lack certain other advantages. High copy number and temperature sensitivity are also useful for some experiments. Also, a wide host range can be very important, but probably only if transfer is non-self-transmissible.

2 General Protocol

The primary focus of the work is a systematic screening among soil bacteria for plasmids coding for resistance to antibiotics. The analysis will follow several lines.

1) Selection of resistant bacteria, followed by rapid characterization of the antibiotic-resistance phenotype by in vitro disc assay [16][17]. Multiply-resistant strains will be further tested. This phase

of the analysis will use a computer program to evaluate the raw data, point out strain similarities, and sort resistance phenotypes.

2) Extraction of DNA, followed by further testing of those isolates containing plasmid DNA.

3) Testing for genetic instability in the absence of antibiotic selection. Certain plasmid-host combinations are more beneficial than others, and this may be reflected in the frequency with which some classes of R-factors are isolated from naturally-occurring strains [1]. The effects of selection on plasmid maintenance should not be ignored.

4) Transferability to B. subtilis, E. coli, and other hosts. Will the new plasmid be able to replicate and express in a foreign host that is genetically and biochemically well-characterized? An important point here is to distinguish between failure to transfer vs. failure to replicate.

5) Molecular analysis. This includes modern molecular analytical techniques such as restriction enzyme digests to produce a physical map, and electron microscopic heteroduplex analysis among related plasmids to elucidate structural similarities at a very fine resolution.

3 Previous Results

To date, three soil samples have been collected. The first was taken about 100 feet from the summit of Mt. Whitney, in an area usually devoid of human beings. The second was from a local stream-bank (San Francisquito Creek), receiving drainage from near the Stanford Hospital. The third was also from a stream-bank, in Portola Valley, situated far from a hospital setting.

3.1 Methods

All samples (about 100 g) were collected in a sterile beaker after scraping away the top two centimeters of soil. Samples were transported to the laboratory as quickly as possible. [In the case of the Mt. Whitney sample, this was 3 days. For the other samples, it was

less than an hour.] In the lab, a 10% w/w soil solution was made in 1/4-strength Ringer's solution, and vortexed for 15 minutes. Following a previously described method [18], dilutions were done in the same buffer, and plated on both selective (nutrient agar + antibiotics, or minimal agar) and non-selective media (nutrient agar).

After about 2 weeks incubation at room temperature, colonies with distinct morphologies were tested for multiple-resistance. The Mt. Whitney sample was screened by replica-plating onto media containing other antibiotics, while subsequent samples were tested simultaneously by the *in vitro* disc susceptibility assay.

Strains were purified by restreaking onto selective media. DNA preps were done to extract plasmids. The clear lysate method [19] was used, although a quick-screening method [20] was also employed in the latter experiments.

3.2 Mt. Whitney sample

3.2.1 Strain Isolation

This initial sample, in addition to serving as an important substrate for screening, was also used to develop the methodology described in the previous section. I tested for resistance to the following agents (1): Ap (25 ug/ml), Cm (15 ug/ml), Er (5 ug/ml), Km (5 ug/ml), Pe (15 ug/ml), Sm (30 ug/ml), and Tm (5 ug/ml). Viable counts on nutrient agar and the number of colonies on selective media were used to construct the following table of % resistant colonies. All growth was at room temperature (22^{o}C).

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(1) Abbreviations for selective agents are: ampicillin, Ap; chloramphenicol, Cm; erythromycin, Er; kanamycin, Km; methyl violet, MV; sodium chloride, NC; neomycin, Nm; novomycin, No; nystatin, Ny; penicillin, Pe; streptomycin, Sm; trimethoprim, Tm; tetracycline, Te.

Table 1
% resistant colonies (2)

antibiotic	Day 4	Day 7	Day 13
Am	1.8	1.8	5.0
Cm	0.3	0.9	2.8
Er	0.5	0.7	1.9
Km	120	120	120
Pe	1.2	1.3	1.6
Sm	0.3	0.5	0.5
Tm	70	75	73
viables/ml(10^4)	1.9	2.2	2.2

The number of strains isolated on each selective medium and replica plated for multiple resistance can be seen in Table 2. Incubation was done, as before, at room temperature ($22^{o}C$).

Table 2

original plate	Am	Cm	Er	Km	Pe	Sm	Tm
strains tested	3	4	6	1	3	6	4

Seventeen isolates showed multiple resistance, all to Am, Er, Pe, and Tm, within 4 phenotypic groups (3). Not all isolates grew in liquid selective media. These were not further analyzed.

3.2.2 Molecular Analysis of Plasmids

DNA preps were made of strains A121, E121, E122, E123, and T121 (4). Purified plasmid DNA was subjected to restriction enzyme digests in order to generate a physical map. All plasmids appeared identical, to the resolution of the technique, by parallel electrophoresis of

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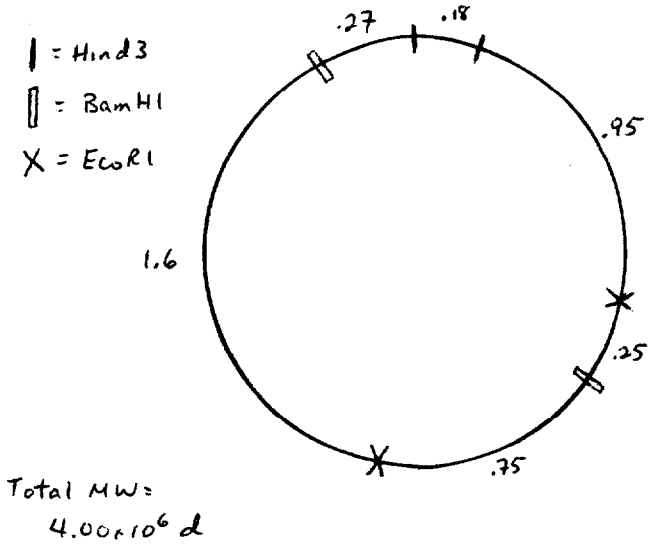
(2) Some of these 'colonies' may have been fungi. However, I attempted to distinguish these from true bacteria by the fuzzyness of the colony morphology.

(3) In addition to $AmErPeTm^r$, there were=> Class 1: 2 isolates were resistant to Sm, Cm, and Km; Class 2: 10 isolates were Cm^r; Class 3: 2 isolates were Km^r; Class 4: 3 isolates with no other resistances.

(4) Strain numbers were determined by using the first letter of the original selective agent, followed by a three digit number. The first digit represents the sample number (1 for Mt. Whitney, 2 for San Francisquito Creek), the second digit is the plate number, and the final digit is the colony number.

single digests through 0.7% agarose gels. More extensive analyses were done by performing double and triple digests on plasmid DNA purified from E121. Enzymes used were EcoR1, BamH1, and Hind3. A unique structure was obtained with a total molecular weight of $4.0*10^6$ daltons. The plasmid map is seen in Figure 1.

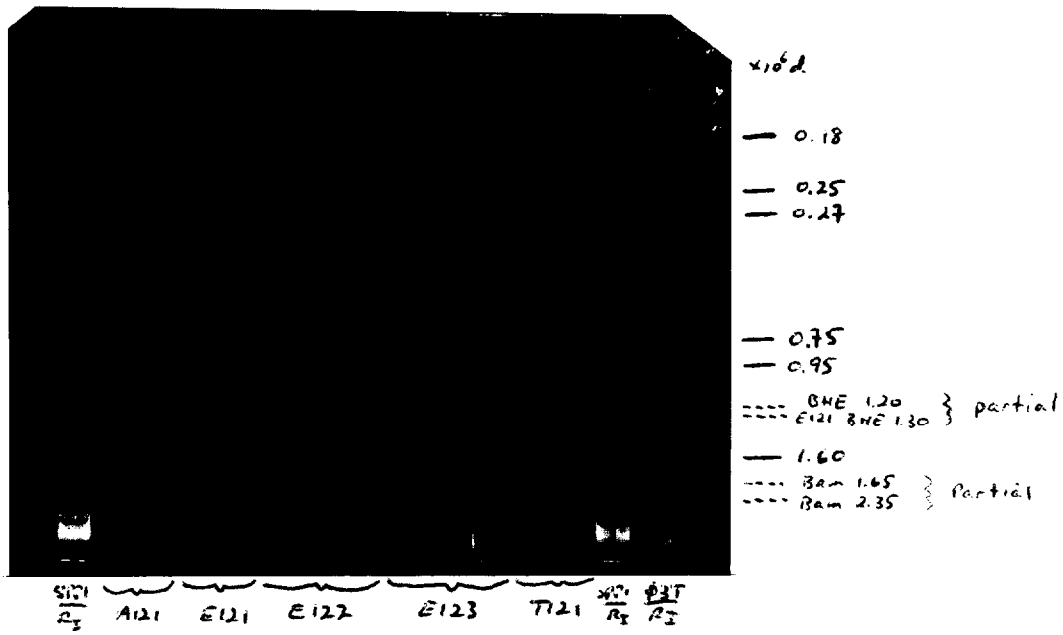
Figure 1



Electron microscopy was done to confirm the total plasmid length. This resulted in a molecular weight determination (4.1+/-0.14 megadaltons) consistent with the estimate of molecular weight based on gels.

To confirm that the plasmids isolated from these strains are identical, I performed triple restriction digests, again using EcoR1, Bam, and Hind3. Plasmids isolated from A121, E121, E122, E123, and T121 all had identical restriction patterns, with one minor exception. A partial digestion product of E121 is about $0.1*10^6$ daltons larger than it's equivalent in all other preps. See Figure 2.

Figure 2



3.2.3 Transformation into E. coli

A strain of E. coli routinely used in our lab was used as a recipient for plasmids isolated from E121, E122, and T121, with pMB9 [21] as a control. After transformation by standard procedures [22], cells were plated on nutrient agar supplemented with Er, Cm, Am, Er+Cm+Am, and nutrient agar alone (for viable counts). Only two colonies were obtained from all the transformation mixtures on selective media. These were both from the mix containing E121 plasmid and selected on Am plates. This represents a frequency of transformation of $2/1.6*10^{8}=1.3*10^{-9}$ or 6 transformants per ug of DNA. The pMB9 control gave a frequency of $2.6*10^{-3}$ of Te^{r} colonies or $1.2*10^{6}$ transformants per ug of DNA. This was 200,000 times more efficient than the E121 Ap^{r} donor.

The two transformants, dubbed A^{r}_{I} and A^{r}_{II}, were replica plated to determine antibiotic resistance and other markers present on the parent strain. They both tested identically, and were Am^{r} Er^{r} Cm^{s} Sm^{r} leu^{-} thy^{-}. The parent strain, W5443, was Sm^{r} leu^{-} thy^{-}. In controls, resistance of W5443 to Am was never seen (less than 10^{-10}). Why was only Am^{r} expressed and it's frequency so low in the E121 DNA transformation?

The most straightforward approach was to purify plasmid DNA from the transformants and compare it to E121 plasmid DNA. This was done for A_I^r and A_{II}^r with identical results. The restriction enzyme patterns between E121 plasmid DNA and plasmid DNA extracted from the E. coli transformants were completely different. The molecular weight of segments generated by digestion with EcoR1, BamH1, and Hind3 had no counterparts between donor and 'recipient' DNAs.

One possible explanation is that the E121 DNA prep contained more than one plasmid. I mapped the smaller one (present in a great molar excess), but small amounts of a large plasmid could also be found. Close examination of the gels did show faint fluorescence near the origin. Another possibility is that a contamination was responsible for the Am^r colonies. A precedent for this can be found in the revised interpretation of the origin of the first plasmid used for genetic engineering experiments, pSC101 [23].

To investigate the source of the plasmid present in A_I^r and A_{II}^r, I looked at DNA homologies between these two plasmids and the E121 plasmid. The Southern technique [24] was employed for this test. Complementary RNA (cRNA) was made against A_I^r and hybridized on nitrocellulose filters to DNA from E121, A_I^r, A_{II}^r, and colE1-amp (as a presumptive negative control). The results clearly showed that A_I^r has sequences common to colE1-amp and not to E121. This was confirmed by the converse experiment: cRNA made against colE1-amp hybridized to A_I^r and A_{II}^r, but not to E121 [25]. These results confirm that the soil plasmids did not go into E. coli and express antibiotic resistance, but rather a contaminant entered which was similar, or identical, to colE1-amp.

3.2.4 Transformation into B. subtilis

Concurrently with the E. coli experiments, I tried to transform highly competent B. subtilis with plasmids purified from the soil bacteria. This series of experiments also gave negative results. Transformation of recipient B. subtilis to resistance to Am, Cm, or Er was less than 10^{-8}.

3.2.5 Attempts to 'cure' -- Elimination of plasmids from isolates

A critical demonstration of the interspecific transfer of plasmids is to return them from the recipient back into the donor strain. This requires that the donor lose it's original plasmids, or be 'cured'. Many clinical isolates spontaneously throw off sensitive variants, while others are almost completely refractory to all attempts at curing ([1],p147). I have found that the Mt. Whitney isolates fall into the latter category.

Checking for spontaneous loss by growth of E122 and T121 in non-selective broth cultures and replica-plating, I found that simultaneous resistance to Am, Cm, and Er was present in 100% of the colonies tested. This represents complete stability for at least 11 generations.

Another reason to attempt to find cured variants is to correlate plasmid DNA with in vivo function. There is no doubt that these isolates are multiply antibiotic resistant. And plasmid DNA can be routinely purified from these strains. There is, however, no evidence that proves that these plasmids code for antibiotic-resistance genes. For this reason, more vigorous efforts were undertaken to employ chemical compounds which will eliminate plasmids from their host bacteria. I used ethidium bromide (EtBr), a potent curing agent for R-factors ([1],pl48) in the first series of experiments. Various concentrations of EtBr in actively growing broth cultures were used, following by replica-plating of survivors. Even with only 5% survival, 100% of the colonies tested were multiply resistant to Am, Cm, and Er. Other agents were used [26], such as sodium dodecyl sulfate, kasugamycin, novomycin, and ultraviolet light. All gave a similar negative result.

3.2.6 Identification

Clearly, in order to state any conclusions about interspecific gene transfer, it is of paramount importance to correctly identify the strains involved. To this end, a collaborative effort was set up with Mr. J. C. Case in our laboratory and Dr. Mortimer Starr at the University of California at Davis to identify the genus and, hopefully, the species of all of the Mt. Whitney isolates.

To date, this has met with only partial success. We have unambiguously determined that all the isolates are pseudomonads, and do not fall into any well-known classification in Bergey's 8th [27] or Stanier's taxonony of aerobic pseudomonads [28]. On the basis of about 30 biochemical and morphological tests, the isolates fall into three phenotypic groups. The difficulty in identification is due mainly to the inadequate taxonomic criteria available at the present time. Many new isolates, in several labs throughout the world, are presenting similar difficulties to the taxonomist of this genus.

At this point, three options are available. First, DNA-RNA homology tests can be performed using the nitrocellulose binding assay [29]. Secondly, DNA melting curves can be done to determine %G-C in the strains. Dr. Manley Mandel at the University of Texas at Houston will be asked to assist us in this regard. Finally, the isolates can be sent to numerical taxonomists specializing in the classification of pseudomonads, for example Dr. B. Holmes at the NCTC in London [30].

3.3 San Francisquito Creek sample

This second sample was collected from soil on the banks of a creek about 1.5 miles from the Stanford University School of Medicine. It was selected in order to check if a much wider range of microorganisms would be found than at the Mt. Whitney site. After suspending the soil, and plating on selective and non-selective media as before, this was indeed found to be the case. Platings were done on nutrient agar alone and supplemented with Am, Cm, Er, Cm+Er, Am+Cm+Er, Km, Cm+Km, Am+Km, Sm, or Te.

Four replicates were made of each plate at each dilution. Colonies were counted on Days 2, 5, 8, and 11. Total viable organisms (e.g. yeasts, bacteria, and actinomycetes) were $(5.1*10^6/ml)*300ml/25g = 6.1*10^7$ per gram of soil. [The Mt. Whitney sample had only about $2.2*10^4$ per gram of soil.] Fungi (as determined by fuzzy colony morphology and rapid spreading) had a density of roughly $5.6*10^4$ per gram. The percent of antibiotic resistant colonies varied from 0.04% for Sm to 0.41% for Km and Am.

Interesting colonies were replica plated on nutrient agar containing Er, Cm+Km, Am+Km, and Tc. Of 53 isolates tested, 7 were yeasts (as shown by phase-contrast microscopy) and 16 were multiply-resistant and grown in nutrient broth to plate for the disc susceptibility tests. Stabs were made immediately for my stock collection. Nine strains were selected for plasmid DNA preps.

Two methods were used to extract plasmids from these strains. No detectable plasmid band could be seen in CsCl-ethidium bromide density gradients using the standard clear lysate method, nor could any plasmids be found in agarose gels by the quick screening method. Thus, although a wider antibiotic resistance spectrum was found, without any evidence of plasmids present in any of the isolates, no further work was done on this sample. Perhaps it will will prove useful as a negative control in future experiments.

4 Current results

4.1 Isolation

The sample with which I am currently working was collected from the banks of another stream, in Portola Valley, rich in humus and plant life. Several methodological improvements were made in the screening of this sample. A potent fungicide, Nystatin, was added to all initial

isolation plate to inhibit the growth of yeasts and fungi. Secondly, plates were incubated at three temperatures (22°, 30°, and 37°C) to increase the variability between isolates. Also, two new non-antibiotic selective agents were added, 5% sodium chloride, and methyl violet (to select for gram-negatives), as well as minimal plates containing only amino acids.

After about one week incubation at their respective temperatures, interesting colonies were streaked on the same selective media, and at the same temperature, as the original plates. They were streak-purified once again, and then transferred on slants and grown in nutrient broth for antibiotic-resistance phenotype determination.

The number of strains being worked with is quite large. Table 3 shows the number of isolates remaining after each purification step.

Table 3

purification step	first streak	second	slant	Ab^r-profile
number of strains	386	295	255	210

4.2 Characterization of isolates

With a collection of 210 isolates, each tested for resistance to 12 antibiotics, a great deal of data is generated. I wrote a computer program, in FORTRAN, to assist in the analysis of the resulting antibiotic-resistance phenotypes. The program takes as input a strain code number, such as A2204 (5), and the diameter of the inhibition zone created by each disc in millimeters.

During program execution, these raw data are converted into phenotypes of Resistant, Intermediate, or Sensitive by programming the standard interpretation chart (supplied with the discs). Percent resistance to each antibiotic is calculated. There is a complete cross-correlation matrix produced, indicating phenotype similarities between all isolates, which assists in detecting identical or nearly identical phenotypes even when the strains are isolated on different media. Finally, a sorted list of resistance phenotypes (ignoring intermediate or sensitive responses) is output.

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(5) This sample, because of the different incubation temperatures, is numbered differently than the previous two samples. The first letter still stands for the selective agent used in the original isolation plate, but the next 2 digits represent the temperature, while the final 2 digits stand for the colony number from that source. Thus, the example given is the fourth colony isolated on Am plates at 22°C.

4.3 Plasmid purification

The most multiply-resistant strains have been selected for plasmid isolation. Two plasmid preps are being simultaneously prepared. The first is a ^{3}H-thymidine-labelled method described for Streptomyces coelicolor [31]. This method was specifically designed to detect covalently closed supercoiled plasmid DNA in an actinomycete. The other method is a large-scale unlabelled prep in a similar growth medium. Having both labelled and unlabelled DNA from actinomycetes will facilitate the design of experiments to screen for DNA homologies between the actinomycetes and R-factor-bearing bacteria in this new sample.

Concurrent with DNA preps are preliminary identification steps including gram stains, phase contrast microscopy, oxidative-fermentative determination, motility testing, flagellar staining, and an oxidase test.

5 Future prospects

The original finding in the Mt. Whitney isolate of identical plasmids in distinct species was quite intriguing. Since the curing experiments have all failed, there is virtually no evidence for specific genetic function of these plasmids. They may be indispensible for the host, such that curing is lethal. Or the appropriate curing agent has not been found. This can be pushed harder by a more focussed and intensive search for antibiotic-sensitive variants using a wide battery of curing agents. However, if the plasmid is present in a very high copy number, it may be impossible to completely eliminate all plasmids from a single organism. If this proves to be the case, it will be very difficult to say anything definitive about the molecular biology of this situation.

Also unsuccessful was the transfer of antibiotic-resistance to E. coli or B. subtilis. The two E. coli transformants turned out to be an artifact after extensive testing. Mixed culture experiments to test for conjugation (data not shown), as well at the transformation experiments described in Section 3.2.3 gave consistently negative results.

On the other hand, the new sample is very promising. There is a very rich diversity of forms, both eubacteria and actinomycetes. The stock currently consists of almost 50 independent actinomycete isolates, as well as about 150 isolates of other types. It is hoped that many plasmids will be discovered, and characterization will rapidly ensue. Our working hypothesis is that there should be a

detectable amount of homology at the DNA level between antibiotic-producers and plasmid-bearing resistant strains. A mass screening at the colony level using the in situ Hogness and Grunstein technique [32] will be used for this purpose. Once gross homologies are found, more detailed analysis will be done, essentially following the scenario in Section 2.

A final aspect of the general problem to be addressed is the quantitative determination of antibiotic-resistance in soil samples from many carefully-selected uninhabited sites. This has the flavor of a comparative epidemiological study and may be important in extending the generality of the depth-first study outlined in this progress report.

References

1. Falkow, S. (1975) Infectious Multiple Drug Resistance, (Pion Ltd., London)

2. Datta, N., Hedges, R.W., Shaw, E.J., Sykes, R.P., and M.H. Richmond (1971) J. Bact. 108:1244

3. Anderson, E.S. (1968) Ann. Rev. Microbiol. 22:132

4. Reanney, D. (1976) Bacteriol. Rev. 40:552

5. Stanisich, V.A. and J.M. Ortiz (1976) J. Gen. Microbiol. 94:281

6. Reanney, D. (1977) Brookhaven Symposium, in press

7. Datta, N. and R.W. Hedges (1972) J. Gen. Microbiol. 70:453

8. Ehrlich, S.D. (1977) Proc. Natl. Acad. Sci. USA 74:1680

9. Gardner, P., Smith, D.H., Beer, W., and R.C. Moellering,Jr. (1969) Lancet (ii):774

10. Walker, M.S. and J.B. Walker (1970) J. Biol. Chem. 245:6683

11. Benveniste, R. and J. Davies (1973) Proc. Natl. Acad. Sci. USA 70:2276

12. Courvalin, P., Fiandt, M., and J. Davies (1977) unpublished manuscript

13. White T., personal communication

14. Finland, M. (1971) Bacterial Infections: Changes in their Causative Agents, Trends, and Possible Basis (Springer Verlag, Berlin)

15. Jukes, T.H. (1971) Ann. N.Y. Acad. Sci. 182:362

16. Federal Register (1972) 37:20525

17. Federal Register (1973) 38:2756

18. Gray, T.G.R. and D. Parkinson, eds. (1968) The Ecology of Soil Bacteria (University of Toronto Press, Toronto)

19. Clewell, D.B and D.R. Helinski (1969) Proc. Natl. Acad. Sci USA 62:1159

20. Meyers, A.M., Sanchez, D., Elwell, L.P., and S. Falkow (1976) J. Bact. 127:1529

21. Maniatis, T., Kee, S.G., Efstratiadis, A., and F.C. Kafatos (1976) Cell 8:163

22. Mandel, M. and A. Higa (1970) J. Mol. Biol. 53:159

23. Cohen, S.N. and A.C.Y. Chang (1977) J. Bact. 132:734

24. Southern, E.M. (1975) J. Mol. Biol. 98:503

25. Lomax, M., personal communication

26. Hahn, F.E. (1976) Antibiotics and Chemotherapy 20:196

27. Bergey's Manual of Determinative Bacteriology, eighth edition (1974) (Wilkins & Wilkins, Baltimore)

28. Stanier, R.Y., Palleroni, N.J., and M. Doudoroff (1966) J. Gen. Microbiol. 43:159

29. Gillespie, D. and S. Spiegelman (1965) J. Mol. Biol. 12:829

30. Holmes, B., Owen, R.J., Evans, A., Malnick, H., and W.R. Willcox (1977) Int. J. Syst. Bact. 27:133

31. Bibb, M.J., Freeman, R.F., and D.A. Hopwood (1977) Mol. Gen. Genet. 154:155

32. Grunstein, M. and D.S. Hogness (1975) Proc. Natl. Acad. Sci. USA 72:3961